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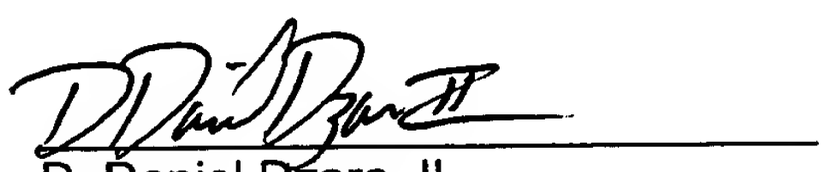

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THE CHARACTERIZATION OF THE DOWNSTREAM BORDER OF THE SUBTILIN OPERON IN *BACILLUS SUBTILIS* LH45 AND AN INVESTIGATION OF THE CELLULAR TARGET OF THE LANTIBIOTIC SUBLANCIN PRODUCED BY *BACILLUS SUBTILIS* 168

by

Virginia W. Rodriguez

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland at College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1999

Advisory Committee:

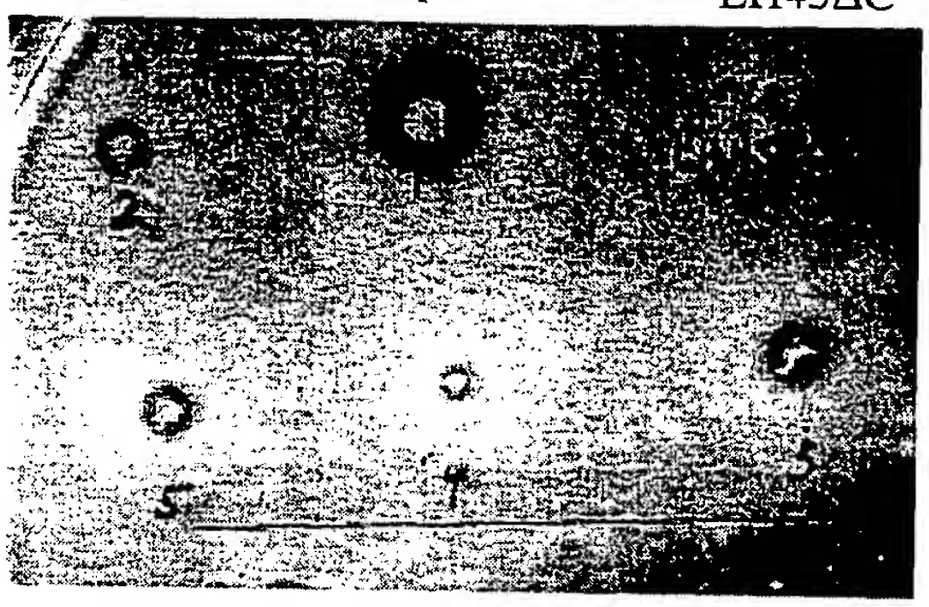
Professor J. Norman Hansen
Assistant Professor Jeffrey Forbes
Associate Professor Douglas Julin
Assistant Professor Jason Kahn
Assistant Professor Richard Stewart

pGR12 . The *B. subtilis* LH45ΔC was transformed with uncut pGR12 DNA. Transformants were selected on PAB plates with 10 μg/ml chloramphenicol. The new strain was designated *B. subtilis* GR12. Five positive clones were toothpicked onto Medium A plates with or without 2mM IPTG. The colonies were incubated at 37°C for 12 hours. The plates were sprayed with *B. cereus* T spores and incubated at 37°C for 4 hours. LH45ΔC was included as a control. This strain has one copy of the subtilin gene under the control of the natural promoter. Clones 3, 4, and 5 had increased activity in the presence of IPTG (Figure 37). Therefore, *B. subtilis* LH45ΔC and clones 3 and 4 were further analyzed for subtilin production by growing 27 hour Medium A cultures with or without IPTG at a final concentration of 2mM. Several attempts were made to purify the subtilin peptide from the cultures using the butanol extraction-acetone precipitation procedure followed by reverse phase HPLC. Clones 3 and 4 were not induced to produce subtilin in the presence of IPTG (Figure 38). It is possible that that the induction of the SPAC promoter led to toxic levels of subtilin, although no evidence of cell lysis was observed. A more likely explanation is that the biosynthesis of subtilin was disrupted. Possibly, the pGR12 plasmid integrated in such a way to disrupt the *spaB* gene sequence so that a non-functional SpaB protein was produced.

3. Construction of Histidine-tagged Subtilin Mutants

In order to simplify the current protein purification procedure for subtilin and subtilin mutants, a histidine epitope tag was placed at different positions in the peptide sequence. The technique of cassette mutagenesis was used to add a

A.



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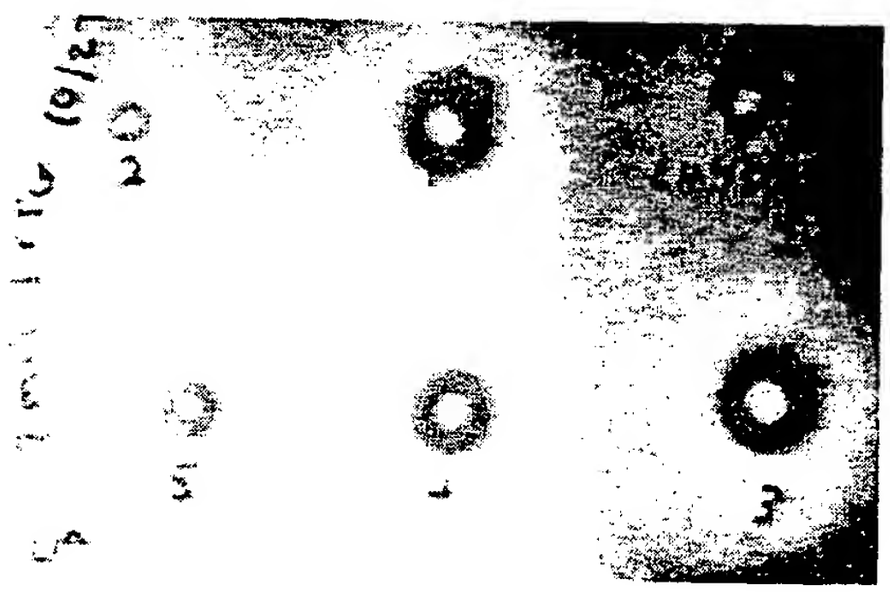
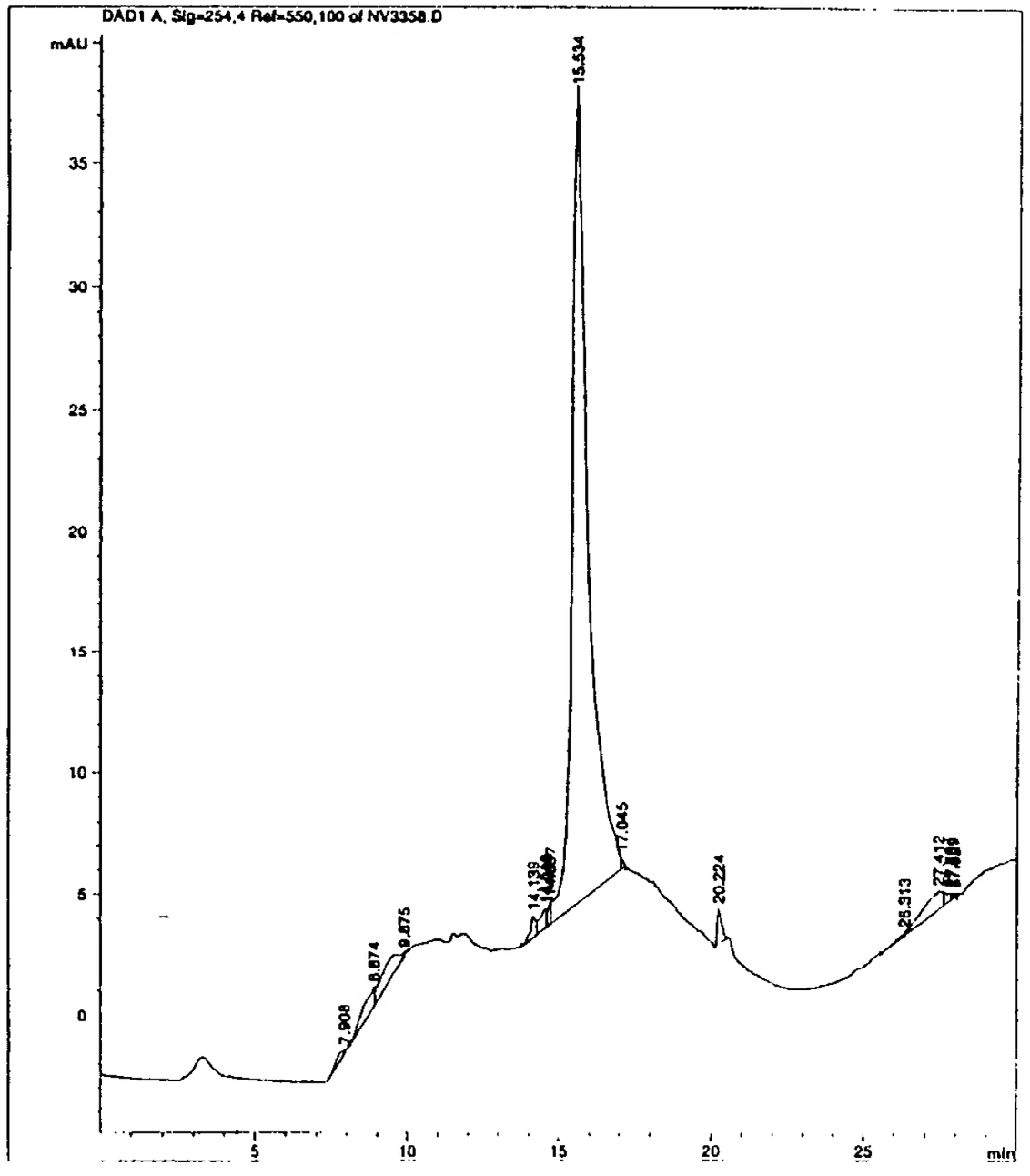


Figure 37. Halo Assay of five clones of *B. subtilis* GR12 and a negative control LH45ΔC in the absence (Panel A) and presence (Panel B) of 2mM IPTG. The five *B. subtilis* GR12 clones have the inducible SPAC promoter in place of the natural promoter of the subtilin operon. LH45ΔC has the nautral promoter of the subtilin operon intact.

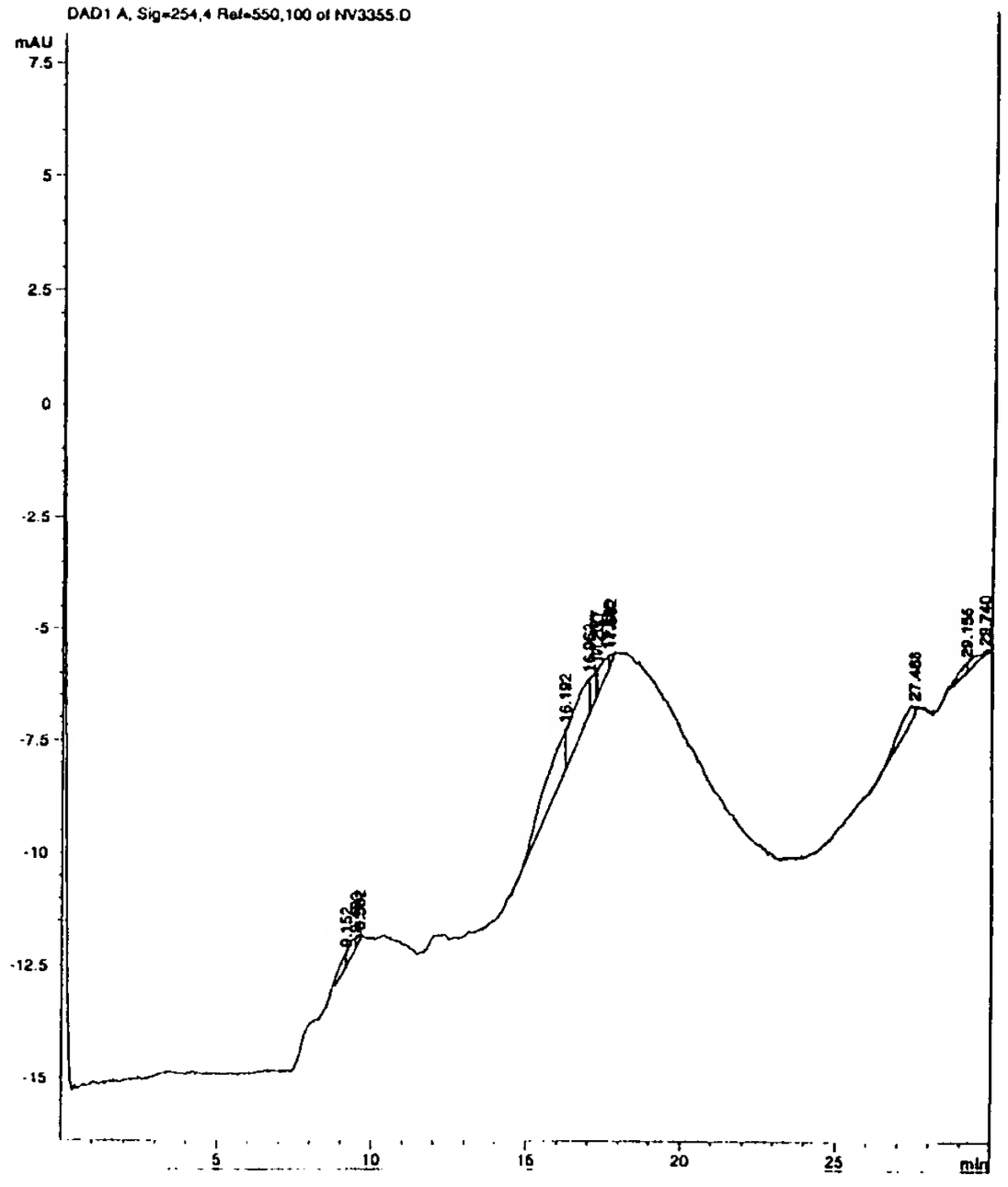
Panel A

B. Subtilis LH45ΔC



Panel B

B. Subtilis GR12
clone 3



Panel C

B. Subtilis GR12

clone 4

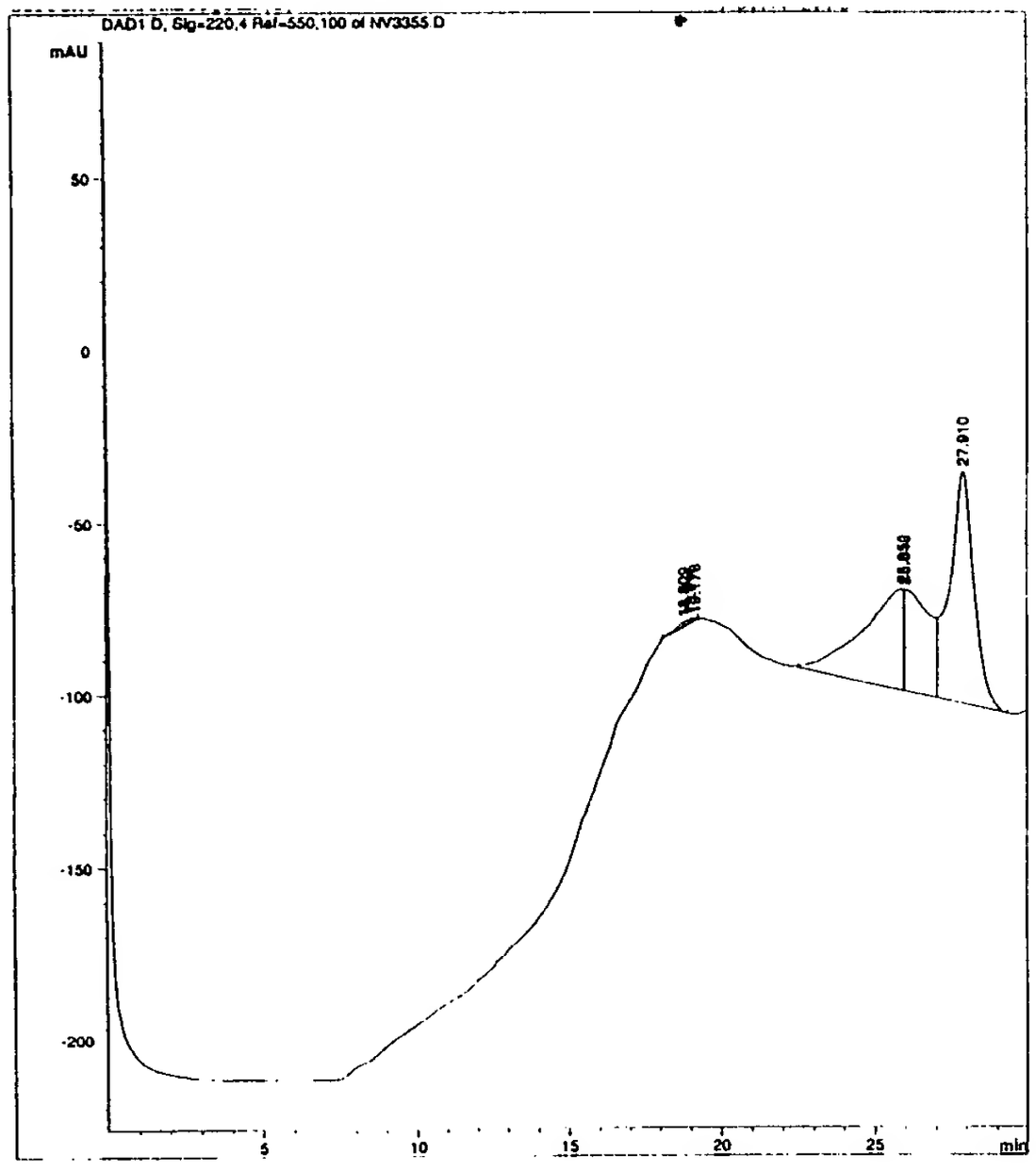
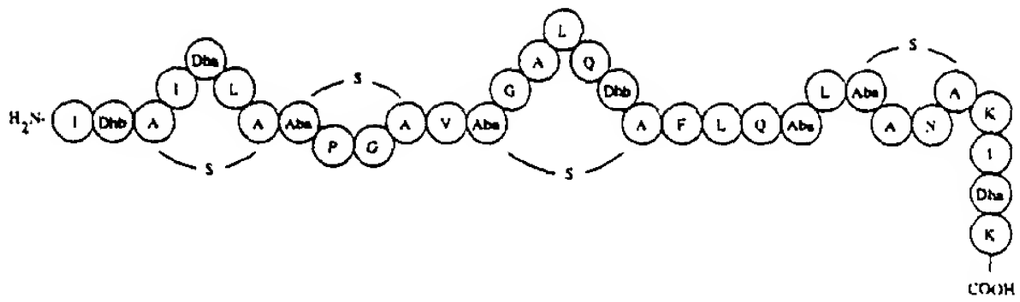


Figure 38. HPLC profiles of subtilin purified from LH45ΔC and from 27 hr Medium A cultures of *B. subtilis* GR12 clone 3 and clone 4 grown in the presence of 2mM IPTG. The subtilin peptide was purified using the butanol extraction - acetone precipitation method followed by reverse HPLC. The absorbance was measured at 254nm to dectect dehydro containing amino acids. *B. subtilis* LH45ΔC is a subtilin producer and contains the natural promoter of the subtilin operon.

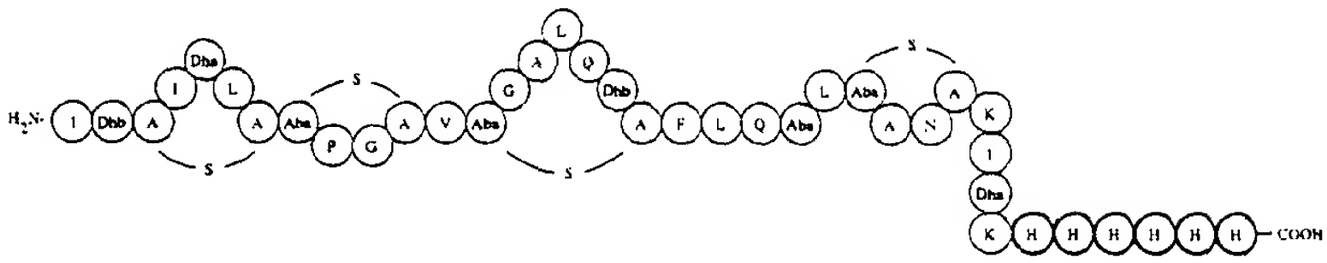
series of six histidine residues at the C-terminal end of the nisin-subtilin chimera and another mutant was made by placing six histidine residues in the flexible hinge region of the nisin$^{1-11}$-subtilin$^{12-32}$ chimera (Figure 39). The chimeric form of subtilin was chosen because it has greater stability compared to the natural subtilin peptide (Chakicherla, 1997). The existing mutagenesis vector pMP3 is a derivative of the pSMcat. The only difference is that the gene for subtilin is replaced with the nisin-subtilin chimera. One problem with the pMP3 plasmid was that it contained two BstEIi sites. It was necessary to use the BstEII site located downstream from the chimeric subtilin gene; therefore, the upstream BstEII was removed. The 4.0 kb BstBI-EcoRI fragment of pMP3 was ligated to the 2.5 kb BstBI-EcoRI fragment of pACcat to generate pMP3a (Figure 40). The plasmid pACcat is also a derivative pSMcat with only one BstEII site, which is located downstream from the subtilin structural gene. The resulting pMP3a plasmid now had only one BstEII site and carried the gene for the nisin subtilin chimera.

Cassette mutagenesis was used to alter the chimeric gene so that it would have a series of six histidine residues added to the C-terminal end or six histidine residues placed in the flexible hinge region between the residues 19 through 23. The details of the mutagenesis reaction as described in Methods, section 20. The inserts containing the mutated genes were each cloned into PTZ19U and positive clones were identified using colony hybridization with the radiolabeled L-1 probe. Plasmid DNA was prepared from several positive clones and this DNA was digested with SmaI and BstEII to release the inserts. The inserts were cloned into

Chimeric Subtilin



Chimeric Subtilin with C-terminal histidine epitope tag



Chimeric Subtilin with histidine tag placed in flexible region

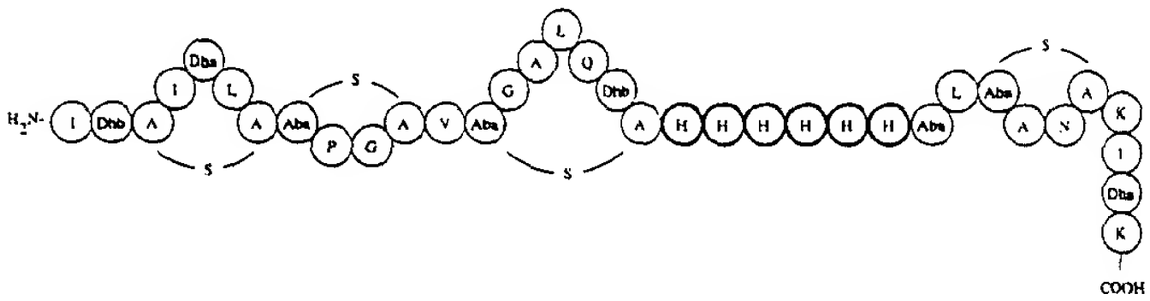


Figure 39. Schematic represenation of the peptide structure of the subtilin chimera and the position of the histidine tag (in bold) placed at the C-terminal end and in the flexible region.

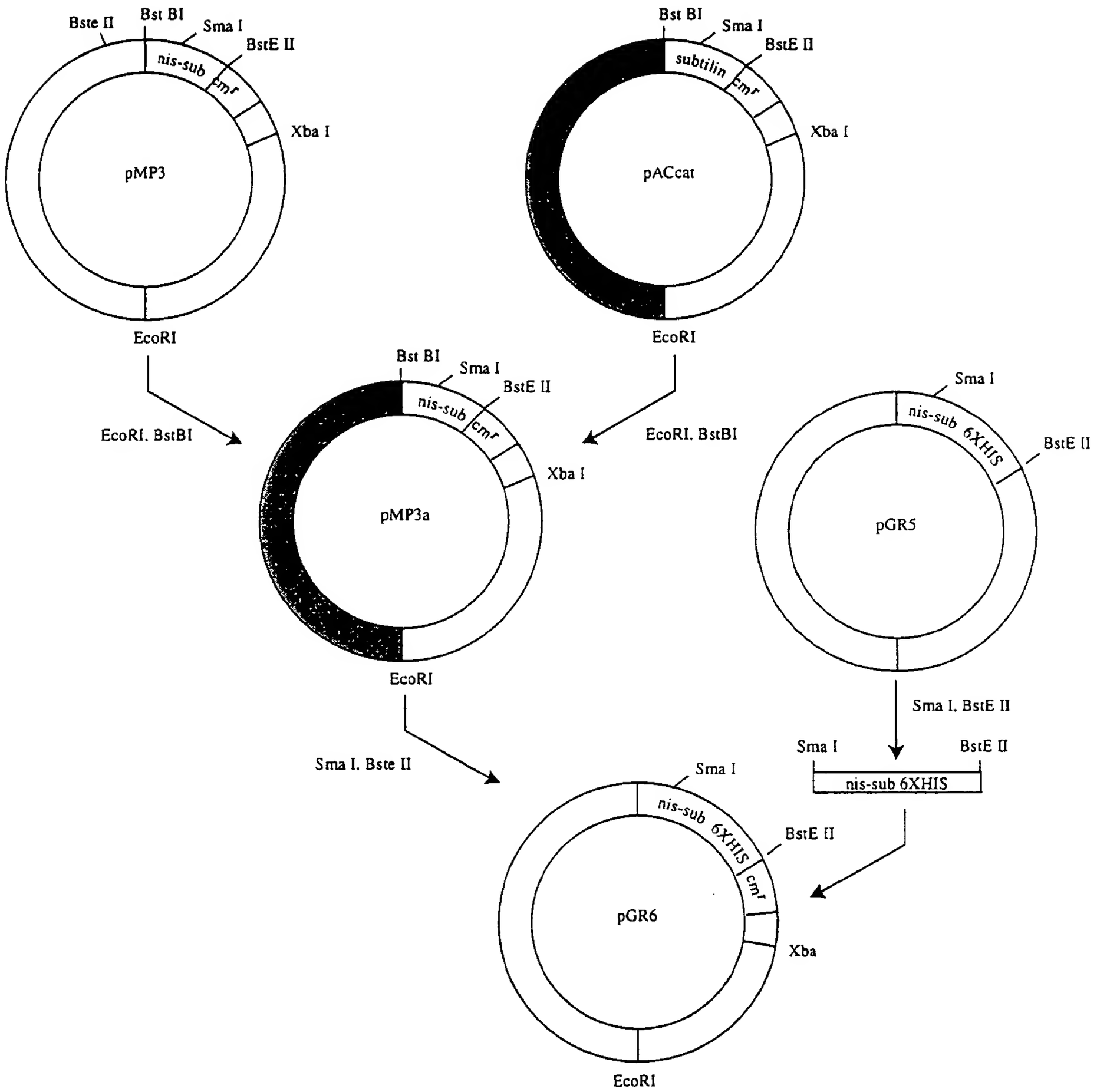


Figure 40. Construction of pGR6. The 4.0kb BstBI - EcoRI fragment of pMP3 was ligated to the 2.5 kb BstBI-EcoRi fragment of pACat to eliminate the upstream BstE II site and insert the nisin - subtilin chimera into the place of subtilin to create the plasmid pMP3a. The Sma I - BstE II fragment carrying the histidine tag from pGR5 cloned into pMP3a to create the plasmid pGR6.

the pMP3a vector, which had also been digested with the restriction enzymes SmaI and BstEII and the resulting plasmid was named pGR6 (C-term 6XHIS) and pGR7 (flex region 6XHIS). Positive clones were identified by restriction analysis. Large scale plasmid DNA was prepared for double stranded DNA sequencing (Methods, section 19.4). The DNA was sequenced using the AN8 oligonucleotide as the primer. The AN8 oligo is a 24 mer that binds to the DNA sequence just upstream of the ribosome binding site located in front of the subtilin structural sequence (Chakicherla, 1997). The sequence for AN8 is given in Table 7 (Methods, section 20.1). The plasmids pGR6 and pGR7 were sequenced using the dideoxy sequencing reaction and they carried the correct histidine mutation.

Once the histidine mutations were verified by sequencing, the plasmid DNA was transferred to the *B. subtilis* LH45ermΔS strain by integrating the plasmid into the chromosome by homologous recombination (Figure 41). The LH45ermΔS strain has the erythromycin resistance gene in place of the subtilin structural gene (Liu, 1992). The plasmid DNA was linearized with the restriction enzyme EcoRI and gel purified. The *B. subtilis* LH45ermΔS strain was transformed with the linearized DNA using the protocol described in Methods, section 17.2. Controls included competent cells only, uncut plasmid PMK4 that replicates in *B. subtilis*, uncut pMP3a and linearized pMP3a plasmid DNA.

After many attempts, transformants arising from double crossover events were never obtained. Individual colonies were screened by growing them on PAB plates containing choramphenicol or erythromycin. This is a way to distinguish between transformants that are a result of homologous recombination

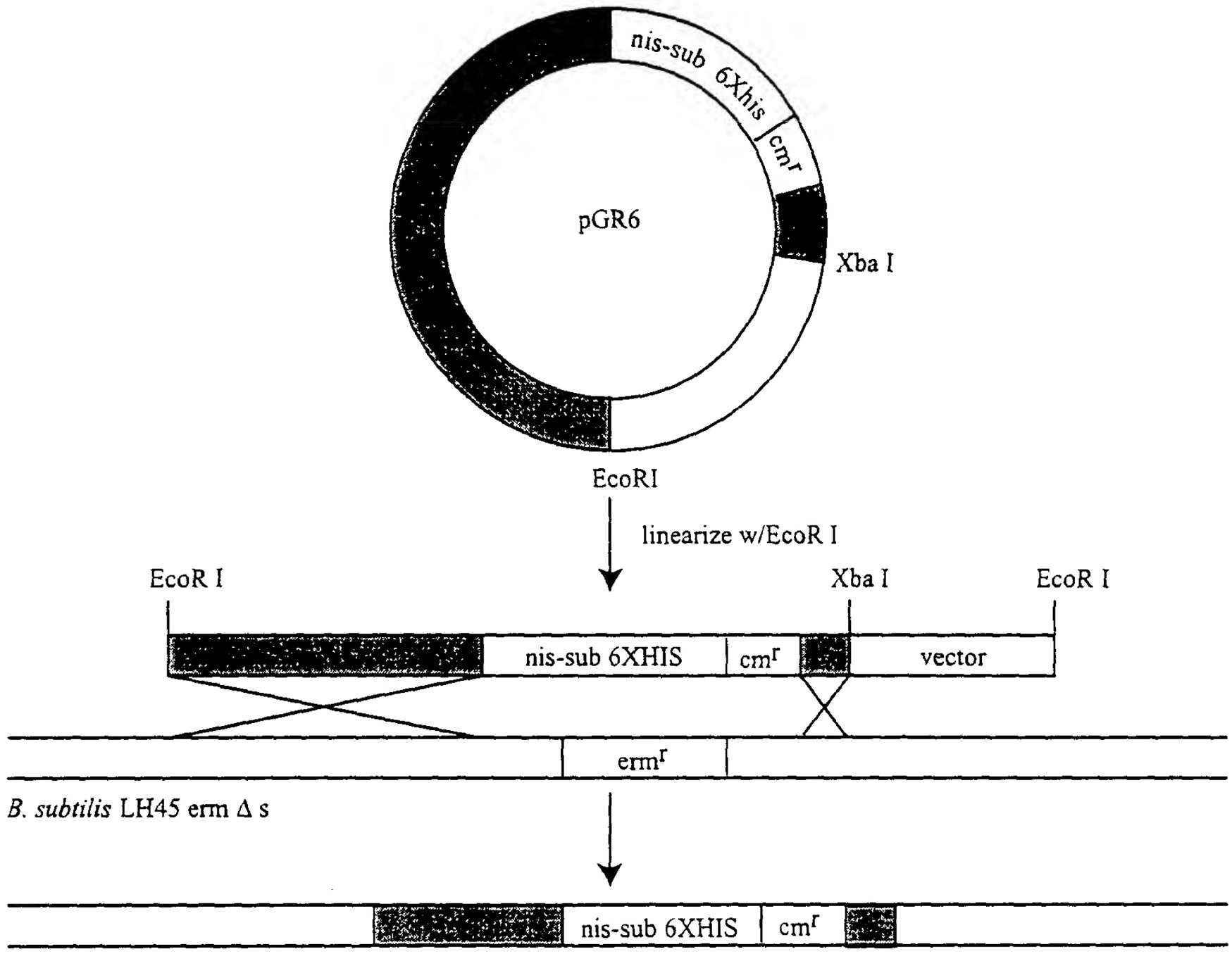


Figure 41. An attempt to create *B.subtilis* GR6 was made but no mutants were obtained as a result of homologous recombination at two separate points due to the small region of homology located after the chloramphenicol resistance gene.

at a single point or at two separate points. A colony that can survive on both PAB/cm and PAB/erm plates is a result of a single crossover event. This type of transformant may not have the subtilin mutant gene in the correct position in the subtilin operon and peptide processing may be disrupted. It is important to select only colonies that grow on PAB/cm plates but not PAB/erm. These transformants will have the erythromycin resistant gene replaced by the subtilin mutant gene and the chloramphenicol resistance gene as a result of homologous recombination at two separate points. This ensures that the subtilin mutant gene is in the correct position in the subtilin operon. Because the positive control (linearized pMP3a plasmid DNA) never produced any double crossovers, the vector was redesigned. The lack of double crossovers could be due to a small region of homology located downstream from the chloramphenicol resistance gene in pGR6 (Figure 41). To increase the region of homology, the 6.3 kb XbaI-SalI fragment of pGR1 was cloned into the XbaI and SalI sites of the original subtilin mutagenesis vector pSMcat (Figure 42). This created the vector pGRcat. Increasing the region of homologous DNA on either side of the subtilin gene should increase the chance of a double crossover event to insert the subtilin gene (or subtilin mutant) into the *B. subtilis* LH45ermΔS strain. The pGRcat was linearized with SalI, gel purified, and transformed into LH45ermΔS. Thirty of three hundred colonies were double crossovers because they were resistant to cholamphenicol and sensitive to erythromycin. Five of the double crossovers were further analyzed using restriction digests to determine if the plasmid DNA correctly integrated into the chromosomal DNA. Genomic DNA was prepared from five of the clones and

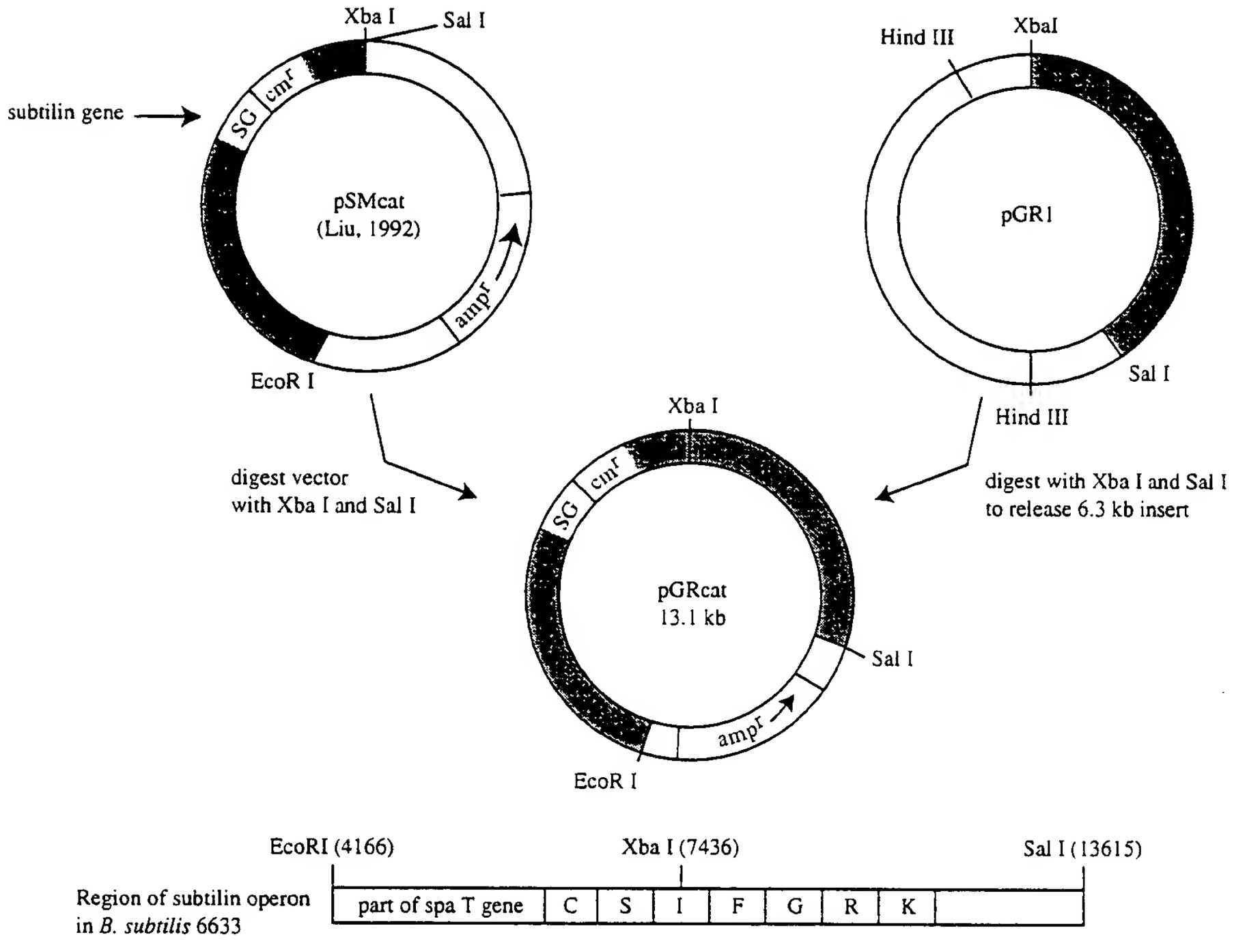


Figure 42. The plasmid pGRcat was created to extend the region of homology downstream (shaded) from the chloramphenicol resistance gene. The diagram of part of the subtilin operon in *B.subtilis* 6633 shows the locations of the EcoRI, Xba I, and the Sal I restriction sites.

digested with several restriction enzymes (Figure 43). All of the pGRcat clones gave the predicted restriction pattern. The predicted 4.1 kb fragment generated by the restriction enzymes PstI and NcoI was present in all pGRcat clones, indicating that the subtilin gene and chloramphenicol resistance gene integrated into the correct position in the chromosome (Figure 43, lanes 2,7,9,11, and13). Also, the *cat* gene replaced the erythromycin gene because when the DNA was digested with NcoI (which cuts only in the *cat* gene), the correct 8.8 kb fragment was generated (Figure 43, lanes 4,8,10,12, and15). One pGRcat clone was chosen and was analyzed for subtilin production by growing a 27 hour culture in Medium A and purifying the subtilin using the butanol extraction-acetone precipitation procedure, followed by reverse phase HPLC (Figure 44).

A halo assay indicated that the purified subtilin from *B. subtilis* GRcat had activity against *B. cereus* T spores (Figure 45). By using the pGRcat vector, the subtilin gene integrated into the LH45ermΔS strain by homologous recombination at two separate points, inserting the subtilin gene in the correct position in the subtilin operon. Therefore, the nisin-subtilin histidine mutant genes were cloned into the pGRcat vector. The SmaI-XbaI fragments of pGR6 and pGR7 were cloned into the SmaI and XbaI sites of pGRcat, replacing the subtilin gene with the mutant. Correct clones were identified by colony hybridization using radiolabeled probes G1 or G11 that recognize the histidine mutations in the C-terminal end or in the flexible region. The new plasmids were designated pGR8 (C-terminal 6XHIS mutation) and pGR9 (flexible hinge 6XHIS mutation). Plasmid DNA was linearized with the restriction enzyme SalI, gel purified and the

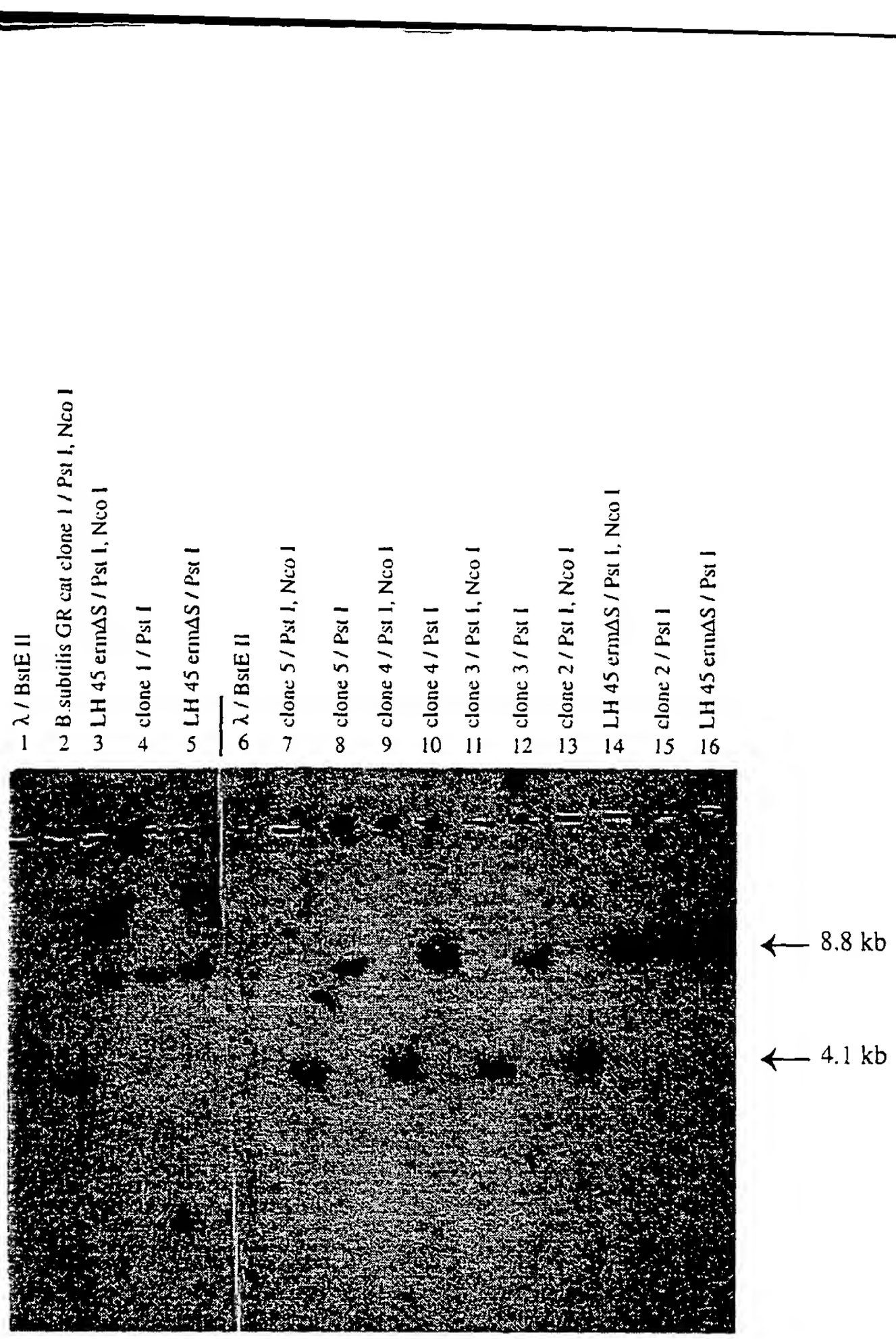
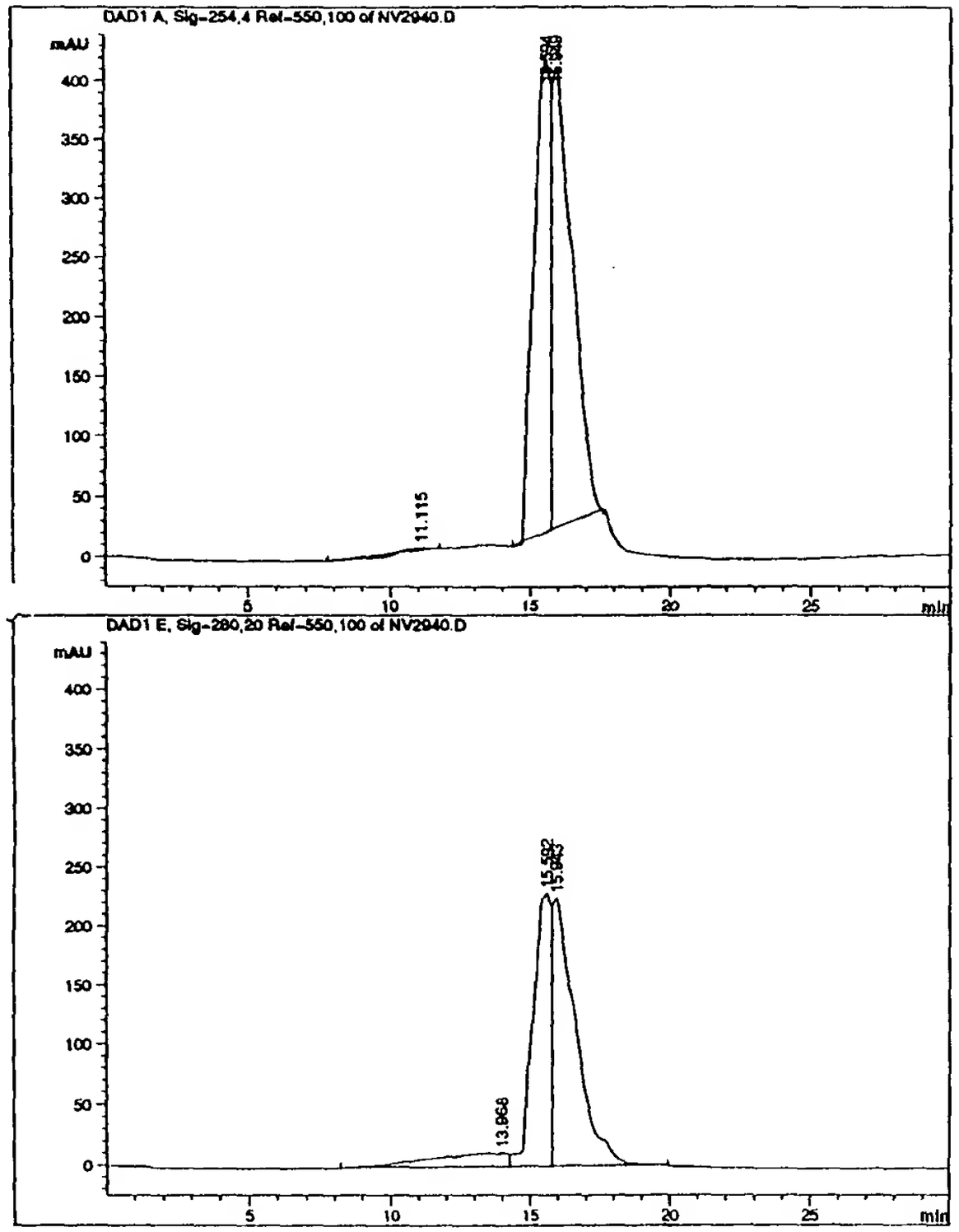


Figure 43. Southern blot of genomic DNA of 5 *B. subtilis* GR cat clones and *B. subtilis* LH45 ermΔS digested with Pst I and Nco I or Pst I probe was used which is described in Table 3. The fragments were the correct sizes as predicted and each clone containing a *cat* gene was cleaved with the restriction enzyme Nco I which is present only in the cat gene and is not in *B. subtilis* LH45 ermΔS.

A



B

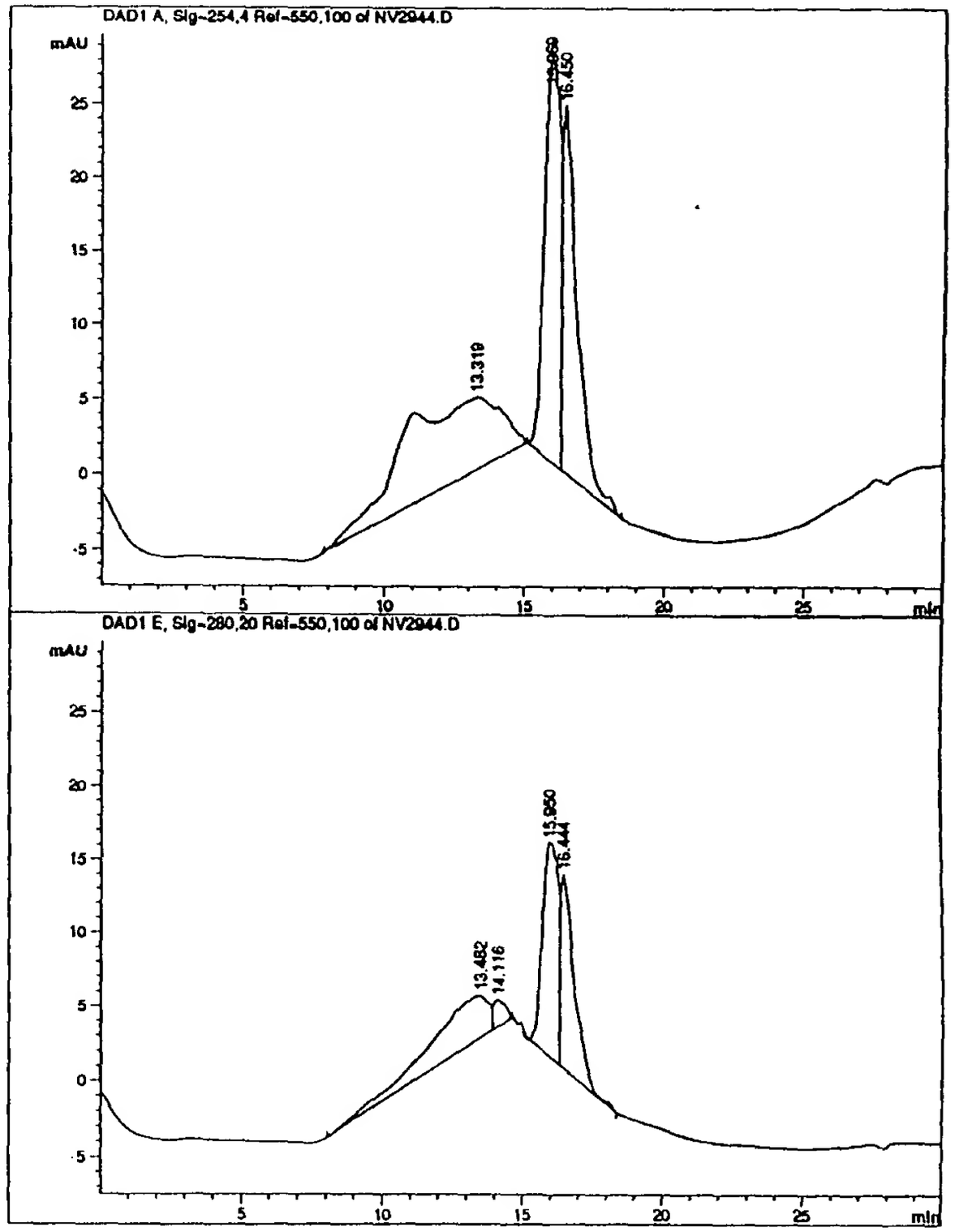


Figure 44. HPLC elution profiles of the subtilin peptide from the subtilin producer *B.subtilis* LH45 (Panel A) and *B.subtilis* GReat (Panel B) from 27 hr medium A cultures purified using the butanol extraction - acetone precipitation methods. The gradient was 0 to 100% acetonitrile in 0.1% TFA for 30 min. The absorbance was monitored at 254nm and 290nm to detect dehydro amino acids and aromatic amino acids.

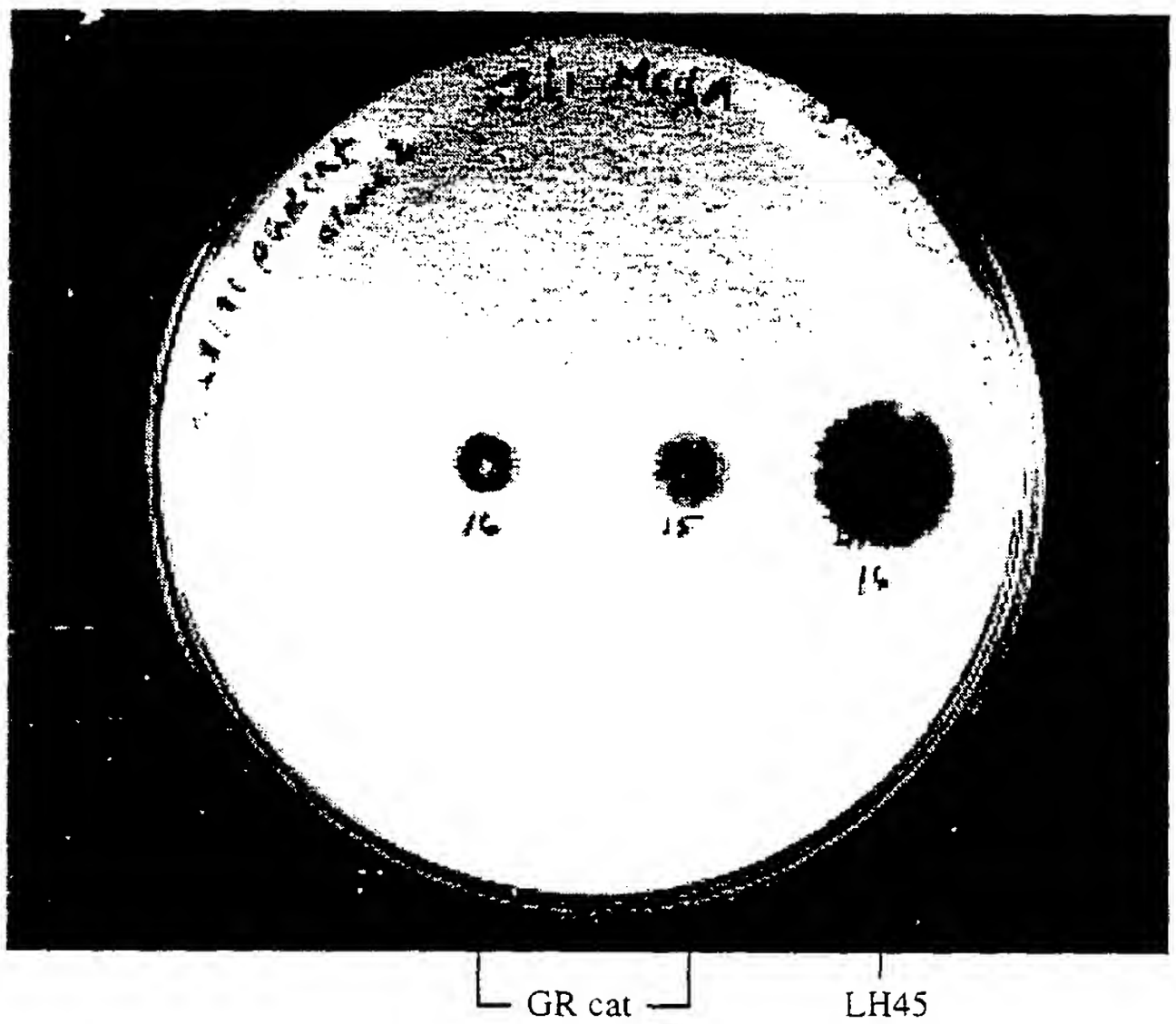
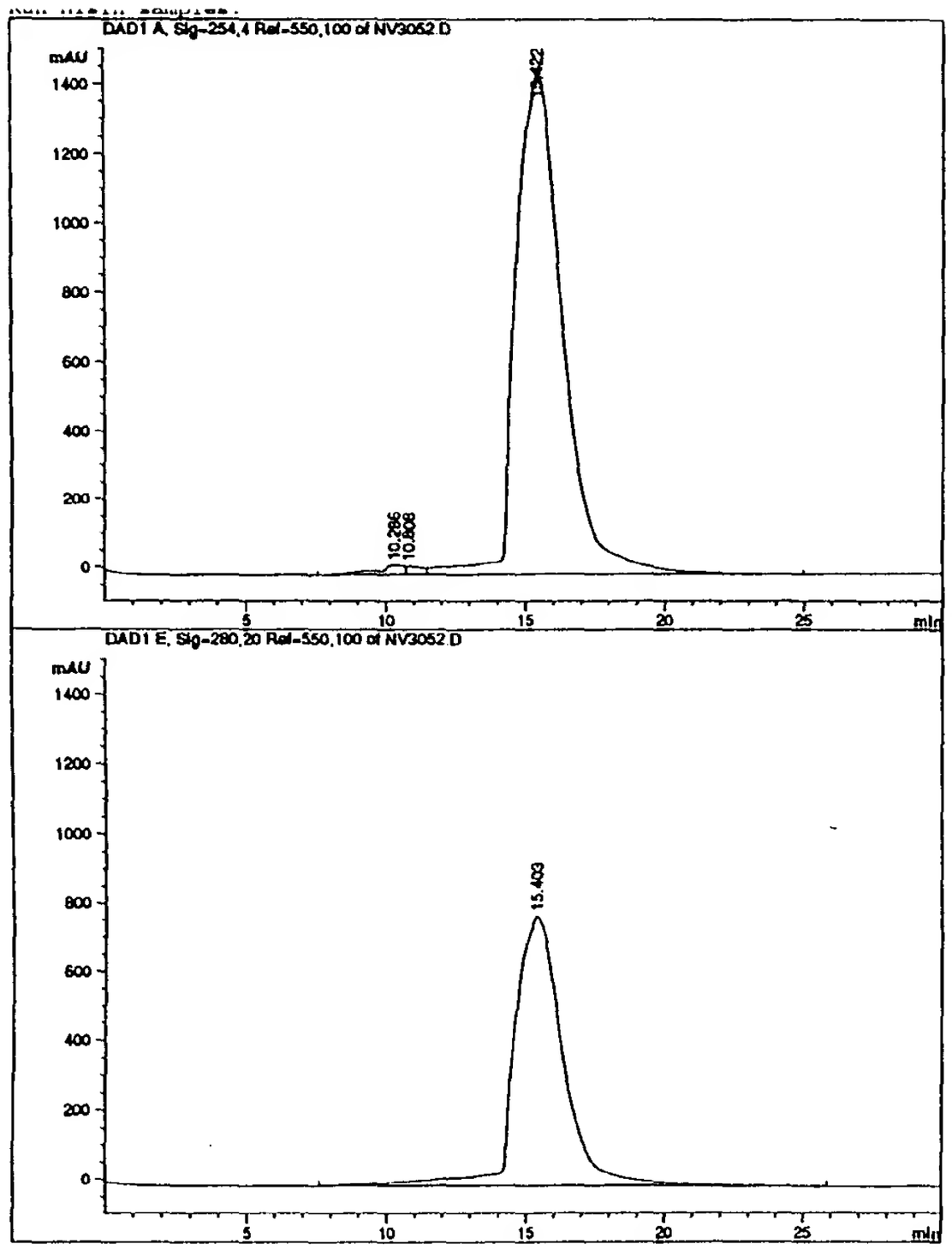


Figure 45. Halo assay of 5 µl of purified subtilin from *B.subtilis* LH45 and *B.subtilis* GRcat purified using reverse phase HPLC. After the samples were spotted on a Medium A plate, it was sprayed with *B.cereus* T spores and incubated overnight at 37° C.

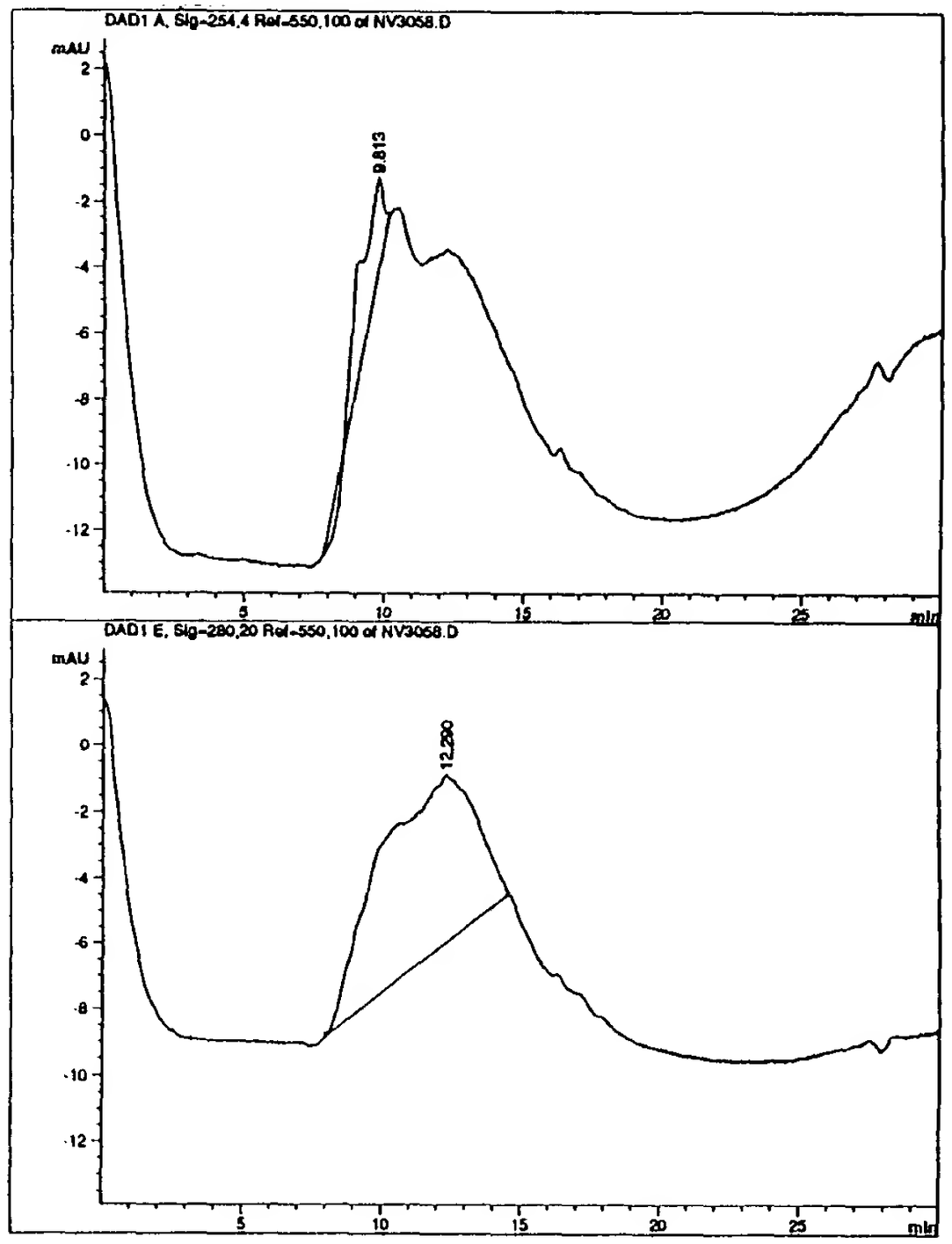
transformations into *B. subtilis* LH45ermΔS were repeated. Double crossovers were obtained for both of the mutations.

Several methods were used to determine if the histidine-tagged subtilin peptides were expressed. The *B. subtilis* LH45 positive control and the histidine mutants were first purified from a 100 ml Medium A culture grown for 27 hours using the butanol extraction-acetone precipitation method, followed by reverse phase HPLC. No chimeric subtilin with a C-terminal histidine tag was produced. However, a possible candidate for the mutant containing a 6XHIS tag in the flexible hinge region eluted at 16.2 min (Figure 46). The elution time for the mutant peptide is later than the control subtilin which eluted at 15.6 min. The lysine and glutamic acid amino acids at positions two and four present in the subtilin peptide are replaced with dehydrobutyrine and isoleucine in the nisin-subtilin chimera, and this would increase the hydrophobic character and cause it to elute later. Because only a small amount of peptide was produced, the entire peak was concentrated by lyophilization and tested for biological activity using a halo assay. It had antimicrobial activity against *B. cereus* T spores (Figure 47). The LH45ermΔS strain which is a negative control because the subtilin gene has been replaced by an erythromycin gene never produced a peptide that eluted after 15 min. The 100 ml culture was scaled up to 1.5 L and the purification procedure was repeated. The amount of peptide did not increase very much, but the sample was active (Figure 48). The theoretical mass of the mutant was calculated to be 3593.7. The mass of the purified peptide as determined by ion spray mass spectroscopy was 3320.47 indicating that the peptide was truncated (Figure 49).

Panel A



Panel B



Panel C

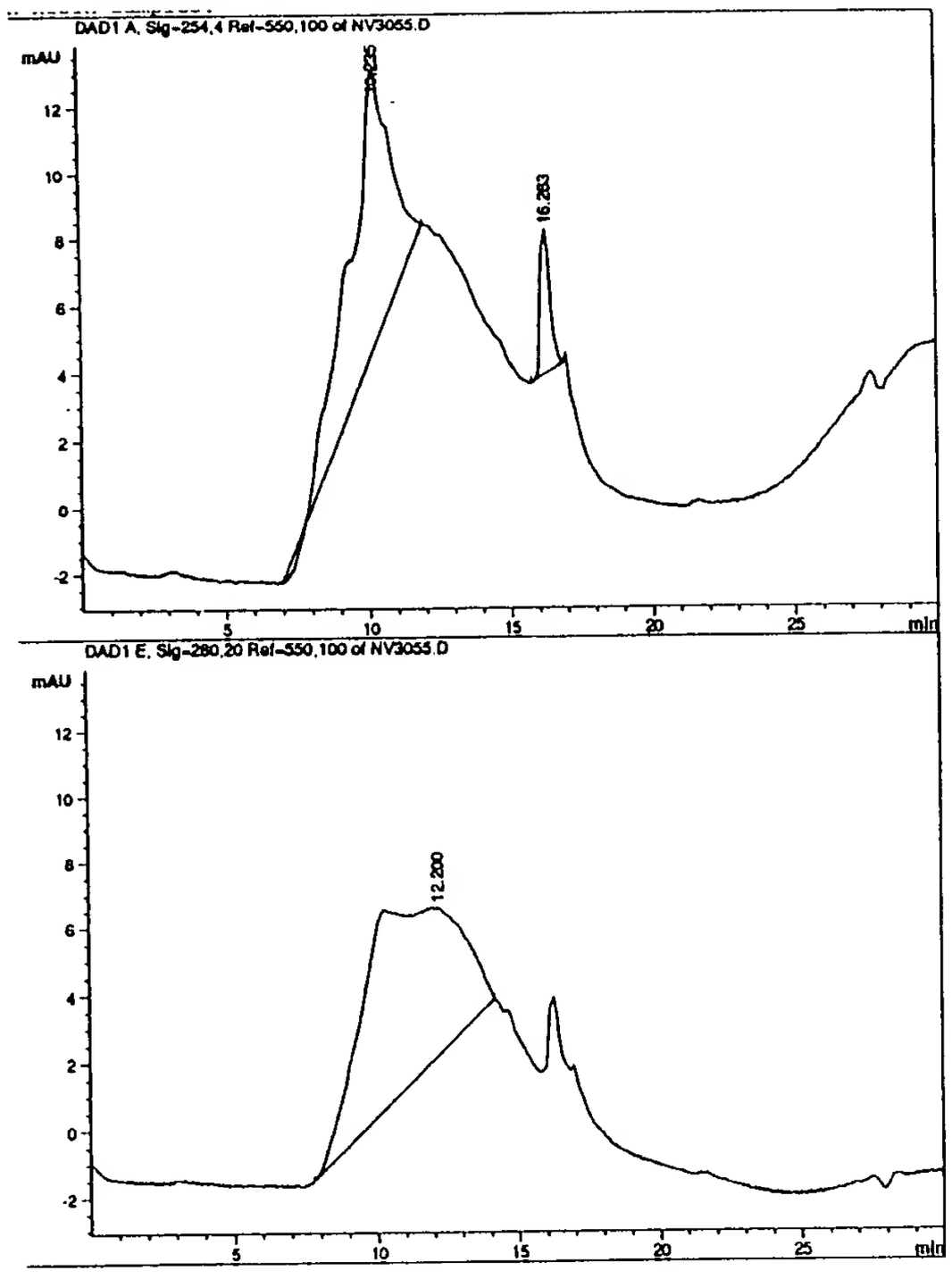


Figure 46. HPLC elution profiles for the subtilin peptide produced by *B. subtilis* LH45 (Panel A) and the C-terminal histidine tagged nisin-subtilin chimera produced by *B. subtilis* GR8 (Panel B) and the nisin-subtilin chimera with a histidine tag in the flexible region produced by *B. subtilis* GR 9 (Panel C). The peptides were purified from 27 hr Medium A cultures using the butanol extraction - acetone precipitation method. The gradient was 0 to 100% acetonitrite in 0.1%TFA for 30 minutes. The subtilin peptide produced by LH45 eluted in 15.4 min (Panel A). The nisin-subtilin chimera with a C-term his. tag was not produced (Panel B) Very little of the nisin-subtilin chimera with a flexible region epitope tag was produced. It was slightly more hydrophobic compared to subtilin and it eluted in 16.2 min(Panel C).

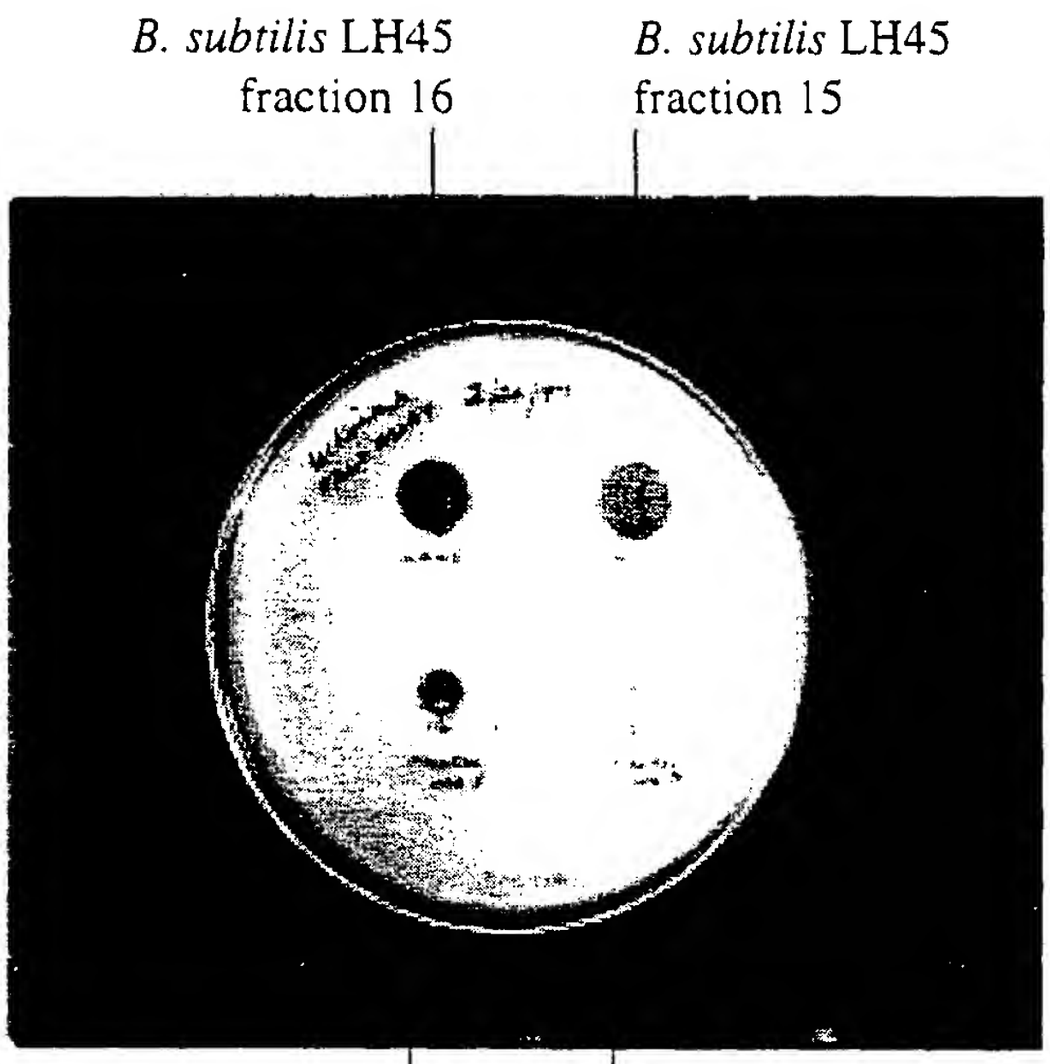
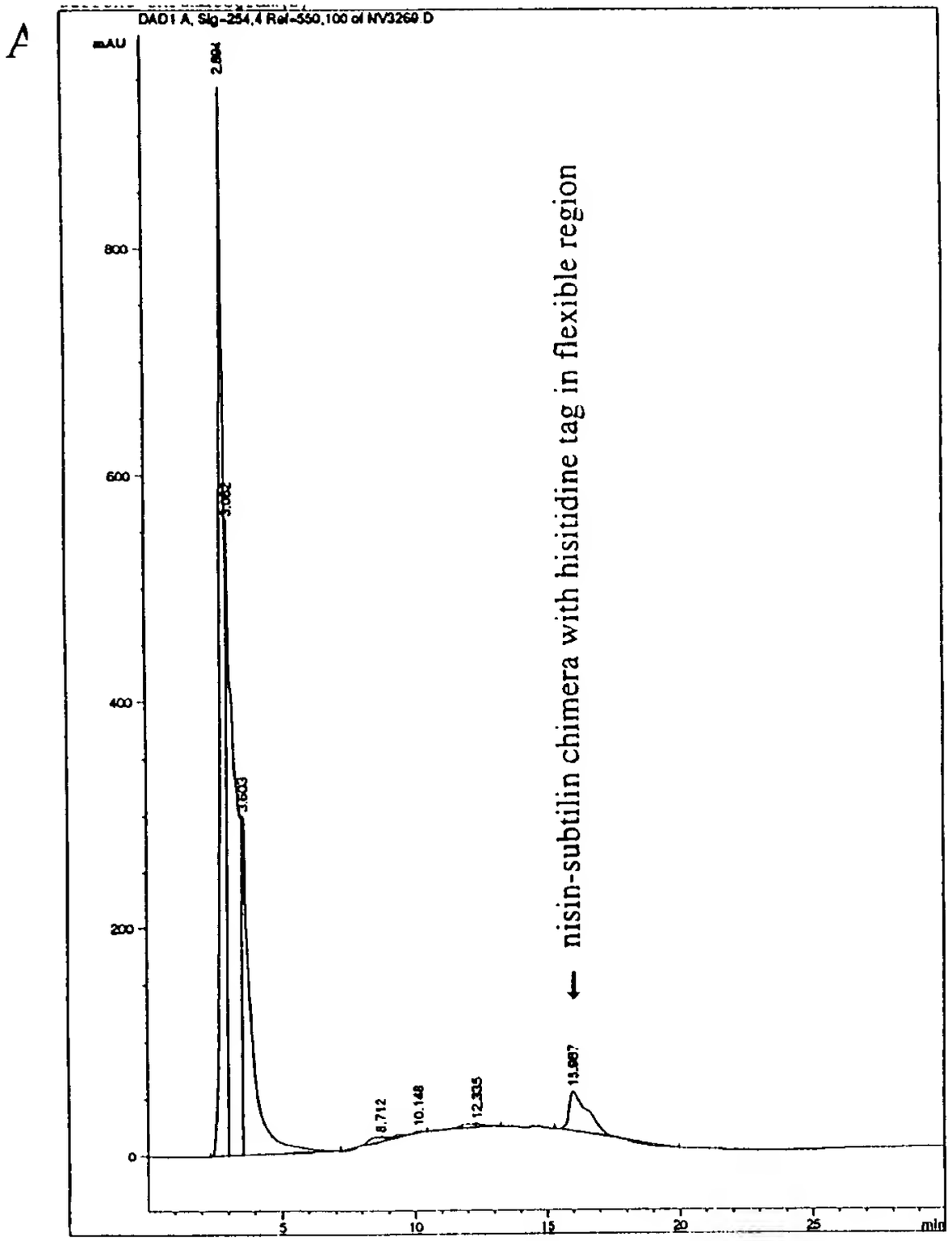


Figure 47. Halo assay of 5 µl of *B.subtilis* LH45 purified subtilin and the entire fractions collected at 15 minutes and 16 minutes for the purified nisin-subtilin chimera with a histidine tag in the flexible region produced by *B. subtilis* GR9. The *B. subtilis* GR9 fractions were concentrated by lyophilization and resuspended in 5 µl of 0.1% TFA. The samples were spotted on a Medium A plate and allowed to air dry and then the plate was sprayed with *B. cereus* T spores and incubated overnight at 37°C



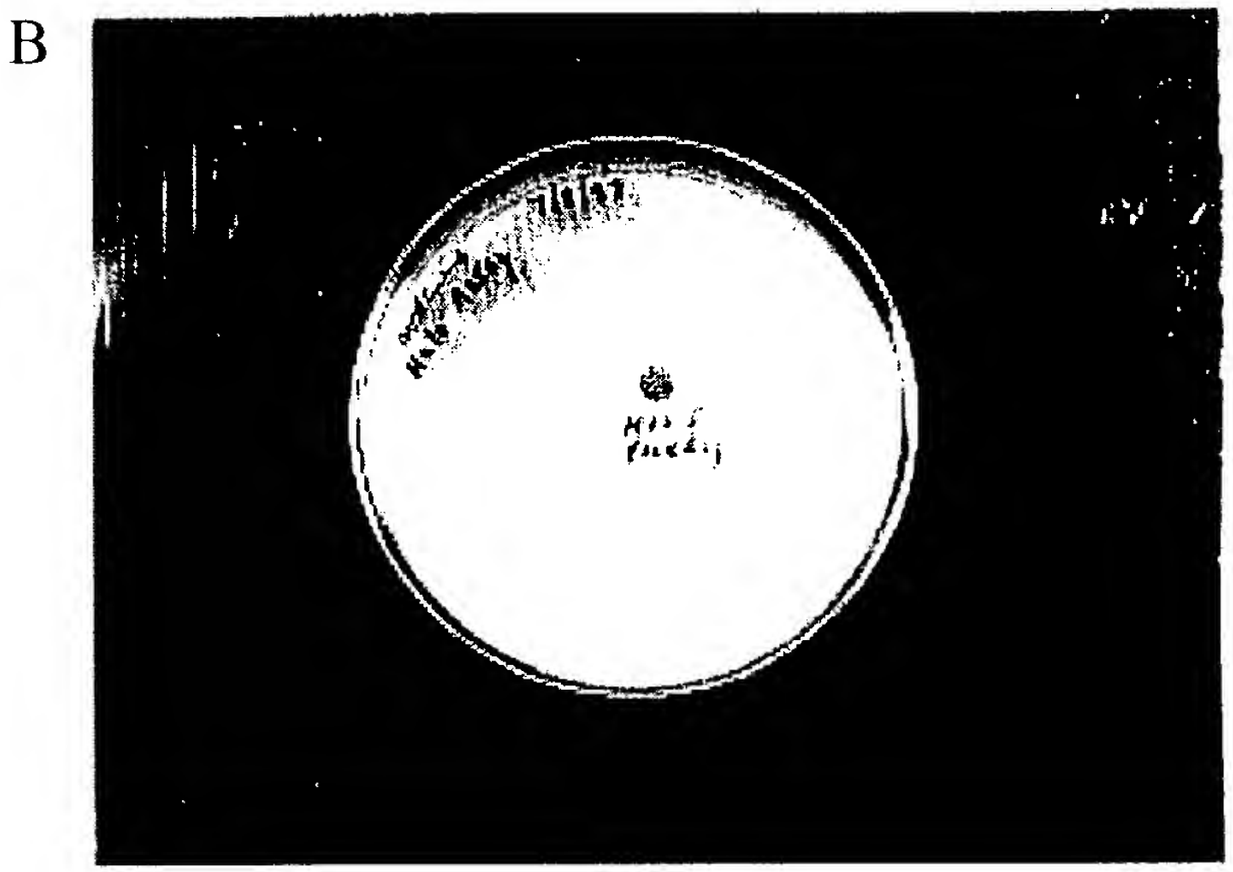
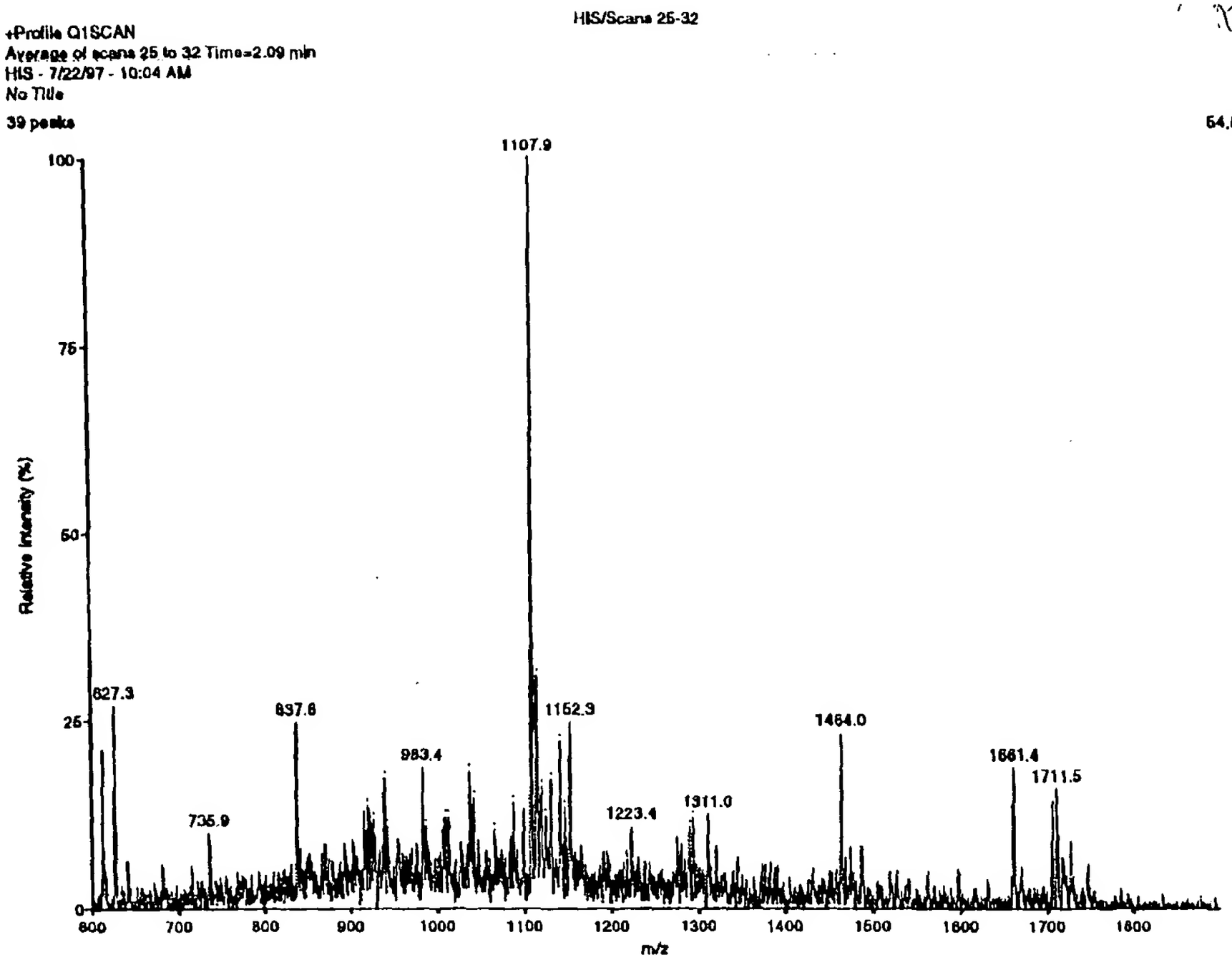


Figure 48. HPLC elution profile (Panel A) and halo assay (Panel B) of the nisin-subtilin chimeric peptide with a histidine tag located in the flexible region. The small peak at 15.9 minutes was analyzed for biological activity by concentrating 75 µl of the 1 ml HPLC fraction and resuspending it in 5 µl of 0.1% TFA and spotting it on a Medium A plate. After the sample was allowed to air dry, it was sprayed with *B. cereus* T spores and incubated over night at 37°C.



Actual peak	Intensity	Pred. peak	Charge	Compound mass
1107.60	54,500	1107.94	3	3319.78
1661.40	10,250	1661.40	2	3320.78

Avg. compound mass 3320.28 Std. Deviation: 0.71
2 Estimates of compound mass

Figure 49. Mass spectrum of the purified nisin-subtilin chimera with a histidine tag located in the flexible region. The theoretical mass for the mutant was caluculated to be 3593.7 which is larger than the observed mass indicating that the mutant ptide is truncated.

Many attempts were made to purify the flexible region mutant, but the *B. subtilis* GR9 cells never produced enough peptide for amino acid analysis. In addition, many attempts were made to purify the histidine mutants using Ni^{2+}-NTA affinity chromatography, but proved to be unsuccessful. Subcellular fractions of the histidine mutants were prepared and analyzed for protein expression using the Western blot technique. There was no evidence of a histidine-tagged peptide in any of the fractions. It is possible that the histidine tag in the flexible region was not recognized by the Ni^{2+} alkaline phophatase conjugate because the epitope was sterically hindered by rings C, D, and E in the tertiary structure of the chimera.

In summary, The six histidine mutation placed at the C-terminal end was so disruptive to the processing pathway that production of the peptide was abolished. The same mutation placed in the flexible region between amino acids 19 and 23 resulted in the production of a peptide at very low levels. The prepeptide was probably recognized by the processing enzymes because the mature peptide had antimicrobial activity against *B. cereus* T spores, which is evidence that the mutant possessed modified residues. The mass of the peptide indicates that the peptide was truncated. Unfortunately, very little of the mutant peptide was produced and there was not enough for amino acid analysis. It is not clear exactly when the peptide was truncated. This may have occurred before it was transported across the membrane or after the leader peptidase removed the leader sequence.